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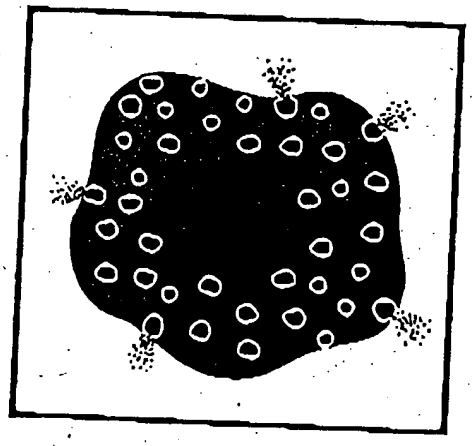

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Review Article

Tissue expression, structure and function of the murine Ly-6 family of molecules

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Summary Murine Ly-6 molecules are a family of cell surface glycoproteins which have interesting patterns of tissue expression during haematopoiesis from multipotential stem cells to lineage committed precursor cells, and on specific leucocyte subpopulations in the peripheral lymphoid tissues. These interesting patterns of tissue expression suggest an intimate association between the regulation of Ly-6 expression and the development and homeostasis of the immune system. Ly-6 molecules are low molecular weight phosphatidyl inositol anchored glycoproteins with remarkable amino acid homology throughout a distinctive cysteine rich protein domain that is associated predominantly with O-linked carbohydrate. These molecules are encoded by multiple tightly linked genes located on Chr. 15 which have conserved geneomic organization. The *in vivo* functions of Ly-6 molecules are not known although *in vitro* studies suggest a role in cellular activation. This review will summarize our understanding of Ly-6 with regard to tissue expression, molecular structure, gene organization and function.

Key words: cell surface molecules, gene family, lymphopoiesis, T cell activation.

Introduction

A key to unravelling the complex pathways of immune physiology has been through the classical serological approach of raising antisera to lymphocytes.[1] These studies have led to the description of the murine 'Ly-6 family of molecules'; a distinctive collection of low molecular weight (12–20 kDa) phosphatidyl inositol anchored cell surface glycoproteins which are encoded by a multiple gene complex on Chr. 15 and are characterized by leucocyte expression. Although the first studies with conventional polyclonal antisera demonstrated separate polymorphic loci (*Ly-6^a* and *Ly-6^b*, or *Thb$^{(high)}$* and *Thb$^{(low)}$*) the immunogenetic simplicity of this region has been redefined by the generation of a large panel of mAb (Table 1) which detect multiple specificities with markedly different tissue distribution, and which are encoded by *Ly-6*-linked genes. Subsequently, a uniform standard of Ly-6 nomenclature has been established by proposing the names Ly-6A.2, Ly-6B.2, Ly-6C.2, Ly-6D.2 and Ly-6E.1 to describe five serologically defined Ly-6-linked antigens.[2] It is important to note that gene cloning studies suggest that Ly-6A.2 and Ly-6E.1 are allelic gene products, thus the terms Ly-6A.2 and Ly-6A.1 or Ly-6E.1 and Ly-6E.2 and the locus name *Ly-6A/E* have been loosely interchanged in Ly-6 literature. Furthermore, the reactivity of anti-Ly-6D.2 mAb with Ly-6A.2 cDNA transfected cells suggests that Ly-6A/E and Ly-6D molecules are identical.[2] At the time of the establishment of Ly-6 nomenclature, the relationships between ThB and Ly-6A/E, Ly-6B, Ly-6C and amino acid sequences were unknown and thus the nomenclature did not extend to the renaming of ThB. Recently this system of nomenclature has been implemented with the isolation of two novel Ly-6 genes, Ly-6G and Ly-6F.1 although, unfortunately, confusing acronyms such as 'TAP' (T cell Activating Protein) and 'Sca-1' (Stem Cell Antigen-1) are still employed in place of the established name of Ly-6A.2. Furthermore, the renaming of ThB as 'Ly-61' and the addition of two separate acronyms for a single molecule, 'TSA-1' (Thymic Shared Antigen-1) and 'Sca-2' (Stem cell antigen-2) have created further confusion for newcomers to this field. In this review the proposed Ly-6 nomenclature has been adopted, although the name 'Ly-6' has been used to describe the family of ThB, Ly-6A/E, Ly-6B, Ly-6C, Ly-6F, Ly-6G and TSA-1 antigens.

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Analysis of Ly-6 tissue expression

Immunogenetics of Ly-6 and ThB expression

The serological analysis of the *Ly-6-Thb* genetic region has identified two separate but tightly linked polymorphisms which encode antithetical structural epitopes and which control the level of cell surface expression. In particular *Ly-6A/E* and *Ly-6C* loci have two alleles, *Ly-6^a* and *Ly-6^b*, which encode the Ly-6.1 and Ly-6.2 phenotypes and *Thb* is polymorphic with two alleles (*Thb^b* and *Thb^l*) which encode high or low levels of cell surface expression respectively. Antithetical specificities encoded by the *Ly-6A/E*

Table 1 Tissue reactivity of Ly-6-specific mAb

Specificity	Con A % + ve	LPS % + ve	T cells % + ve	B cells % + ve	N % + ve	BM % + ve	LN % + ve	Spleen % + ve	Thymus % + ve	Ref
Ly-6A.2										
5041-24.2	NT	NT	90	65	NT	0	90	70	30	3
SK8.106	100	NT	NT	NT	NT	5	70	55	5	4
E13 161-7	NT	NT	NT	NT	NT	7	21	13-50	8-10	5
D7	>90	NT	NT	NT	NT	<10	NT	50-75	<10	6
YE3/19-1	86	77	NT	NT	NT	3	27	4	2	7
34-11-3	NT	NT	NT	NT	NT	10	65	25-60	0-13	8
3A7	100	100	70	0	NT	NT	NT	NT	10	9
Ly-6B.2										
5119-4/7	NT	NT	NT	NT	>9	40-50	0	0	0	10
SK38.86	NT	NT	NT	NT	NT	50-70	5	5	0	4
Ly-6C.2										
34-2-11	NT	NT	NT	NT	NT	45-80	5-30	15-50	0-6	8
SK142.446	40-50	NT	NT	NT	NT	50-70	30-40	20-30	6	4
143-4-2	NT	NT	NT	NT	NT	50	18	10	<1	11
H9/25	NT	NT	NT	NT	NT	30-40	13	13	1-2	12
5075-3.6	NT	NT	NT	NT	NT	45-72	52-70	49-68	<5	13
6C3	NT	NT	15	0	NT	38	10	NT	NT	14
B4B2	17	4	15	NT	NT	30	8	6	1	15
HK1.4	60	NT	NT	NT	NT	45	15	25	<5	16
Ly-6D.2										
5075-19.1	NT	NT	NT	NT	NT	<5	81-95	85-90	90	13
Ly-6E.1										
SK70.94	NT	NT	NT	NT	NT	5	5	5	5	4
HD42	>90	60-70	NT	NT	NT	<5	20-30	2-5	<5	17
YE3/19.1	NT	NT	NT	NT	NT	5	5	5	5	7
D7	>90	>90	10-15	10-15	NT	<10	NT	20-30	<10	6
Ly-6G										
1A8	NT	NT	NT	NT	NT	45-50	0	0	0	18
RB6-8C5	NT	NT	NT	NT	NT	55-65	NT	5	5	19
ThB										
53-9.2	NT	NT	0	NT	NT	20	NT	65	78	20
49-4H	0	100	<5	NT	NT	27	NT	42-50	50-80	21
TSA-1										
MTS-35	NT	NT	1-5	94-96	NT	72-80	NT	NT	75-85	22
E3 81-2	NT	NT	0	NT	NT	12	NT	<1	26	5

BM, bone marrow; LN, lymph node; N, neutrophils; NT, not tested.

and *Ly-6C* genes have been detected serologically and the mechanism of this allelism is controlled by single amino acid substitutions;[2] presently, *Ly-6B* and *Ly-6F* gene products have been detected only in Ly-6.2 and Ly-6.1 strains, respectively, and it is not known whether these genes encode antithetical structural epitopes. Certainly the positive serological reactivity of anti-ThB, anti-Ly-6G and anti-TSA-1 mAb and the observation that *Thb* and *Ly-6G* encode amino acid sequences without allelic differences in Ly-6.1 (ThB^{low}) and Ly-6.2 (ThB^{high}) strains suggest that the structural polymorphism observed at *Ly-6A/E* and *Ly-6C* does not control the expression of all *Ly-6*-linked genes.[18,19,21-23] The polymorphism which controls the level of cell surface expression has been characterized at the *Thb* locus by analysis of the levels of ThB mRNA and cell surface glycoprotein. These studies suggest that the polymorphism is controlled at the level of gene transcription with higher levels of *Thb* transcription leading to higher levels of ThB mRNA and ThB molecules in ThB^{high} (Ly-6.2) strains, compared with ThB^{low}.[23] It is not clear if the polymorphism at *Thb* is associated with the expression of other *Ly-6*-linked genes.

Expression of Ly-6 molecules in peripheral lymphoid tissues

Ly-6 molecules are expressed on fully differentiated cells in specific compartments of peripheral lymphoid tissues including the spleen, lymph nodes and peripheral blood and there is limited expression on non-lymphoid tissues (Table 1). With respect to peripheral cellular expression it is possible to classify Ly-6A/E, Ly-6B, Ly-6C, Ly-6F, Ly-6G, ThB and TSA-1 according to four partially overlapping subfamilies which include molecules with expression on: (i) B cells (also thymocytes); (ii) T cells; (iii) granulocytes; and (iv) non-lymphoid tissues (Fig. 1).

ThB and TSA-1 are members of subfamily I (Fig. 1); both molecules are expressed on B cells from the spleen, lymph nodes and blood and TSA-1 expression is found in

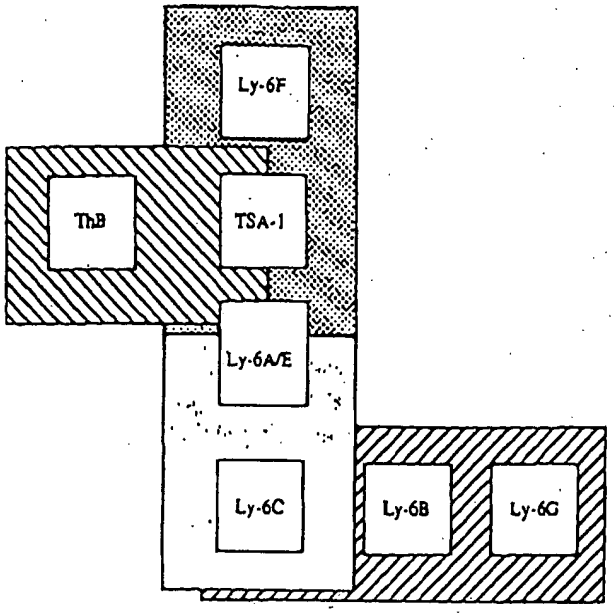

Figure 1 The classification of murine Ly-6 molecules according to patterns of peripheral tissue expression. Subfamily I (ThB and TSA-1; B cells and thymocytes, ▩); Subfamily II (Ly-6A/E and Ly-6C; T cells, ▩); Subfamily III (Ly-6B and Ly-6G; granulocytes, ▨); and Subfamily IV (Ly-6F; non-leucocytes, ▩).

germinal centres in the lymph nodes and spleen.[24] The expression of ThB and TSA-1 in tissue compartments that are associated with lymphoblast transformation (lymph node and spleen germinal centres and also the thymic cortex) suggests a role for these molecules in cellular activation. As defined by subfamily I, expression is also detected on thymocytes, although ThB and TSA-1 are not expressed on peripheral T cells and ThB expression cannot be up-regulated by ConA stimulation of purified T cells.[21]

The Ly-6A/E and Ly-6C antigens are members of subfamily II, with expression particularly on peripheral T cells (Fig. 1). In addition, the Ly-6A/E antigen is expressed on peripheral B cells (thus Ly-6A/E expression partially overlaps with subfamily I) and also on NK cells.[25] Ly-6A/E molecules are expressed in the T cell regions of the splenic white pulp, some regions of the red pulp and there is also expression on basement membranes surrounding the periarteriolar sheath and white pulp marginal zones.[24] There are fewer Ly-6A/E$^+$ cells in Ly-6.1 mice compared with Ly-6.2 mice, although the levels of cellular expression are enhanced on mitogen activated T cells and B cell blasts. Furthermore, Ly-6A/E molecules are upregulated in the response to IFN-γ, IFN-α/β and TNF.[26-29] Ly-6C expression is found exclusively on 40% of the CD8$^+$ peripheral T cells subset and accordingly expression is observed on spleen (10%) and lymph node (18%). In a similar fashion to Ly-6A/E molecules, the expression of Ly-6C molecules is increased on T cells by mitogenic stimulus and IFN-α/β although there is no effect on Ly-6C expression with IFN-γ.[9,26,27]

Subfamily III describes molecules with expression on granulocytes and includes Ly-6B and Ly-6G antigens (Fig. 1). Ly-6B is detected on neutrophils and 5% of lymphocytes and Ly-6G is expressed on neutrophils in the bone marrow and less than 5% of thymus or spleen cells (Table 1). Ly-6C, which is a member of subfamily II, has overlapping expression with subfamily III with expression on macrophages, eosinophils and neutrophils from adult bone marrow.[16]

Although initially defined as leucocyte specific antigens, recent studies suggest that Ly-6 expression may extend to non-lymphoid tissues, as defined by subfamily IV (Fig. 1). Indeed, Ly-6F molecules are expressed solely on non-lymphoid tissues, including testes.[30] TSA-1 (subfamily I) has overlapping expression with subfamily IV, as TSA-1 mRNA is detected in non-lymphoid tissues including testes, muscle, liver, lung, brain and heart. In addition, the expression Ly-6A/E molecules (subfamily II) overlaps with subfamily IV and is detected on endothelial cells in spleen, kidney, heart, liver and brain tissues; expression is also observed on mouse testicular Leydig and Sertoli cells.[31,32] These examples of non-lymphoid expression suggest that the functional roles of TSA-1 and Ly-6A/E molecules are unlikely to be lymphocyte specific and in this context it is interesting to note that ThB molecules represent the only members of subfamily I and II which are not also expressed in subfamily IV. Thus the functional role(s) of ThB molecules are likely to be lymphocyte specific.

Ly-6 expression during haematopoiesis

The pathways of cellular differentiation from stem cells to lineage committed precursors are very complex, although it is generally accepted that the bone marrow microenvironment contains the necessary requirements for the generation of B lymphocyte, granulocyte and myelomonocytic elements; for T cells it is unknown whether stem cells seed the thymus and become lineage committed, or if a pre-commitment event occurs in the bone marrow. Nonetheless, it is clear that the regulation of Ly-6 expression from stem cells to lineage committed precursors in the bone marrow and thymus is intimately involved with cellular differentiation events. These interesting patterns of expression suggest that Ly-6 molecules play an important role in haematopoiesis by interaction with, as yet, undefined ligands.

Ly-6A/E expression on haematopoietic precursors

The observation of Ly-6A/E expression on a haematopoietic stem cell subpopulation is a measure of the intimacy of the relationship between Ly-6 expression and haematopoiesis. Adult murine haematopoietic stem cells are a rare bone marrow subpopulation (0.05–0.3%) that are defined by the expression of Thy-1^{lo}, but with no expression of markers (B220, Ly-6G, Mac-1, CD8) associated with lineage commitment (i.e. the stem cell phenotype is Thy-1^{lo} Lineage$^-$ (Lin$^-$). The Thy-1^{lo} Lin$^-$ population is further divided, phenotypically and functionally, by the expression of Ly-6A/E (Sca-1) molecules on adult bone marrow and fetal liver derived stem cells.[33] Approximately 20–30% of Thy-1^{lo} Lin$^-$ cells are Ly-6A/E$^+$ and this subset contains highly enriched stem cell

activity. Adoptive transfer of as few as 30 of the Ly-6A/E$^+$ Thy-1^{lo} Lin$^-$ cells can rescue lethally irradiated mice and reconstitute all blood cell types in the recipient animal. Thus, the Ly6A/E$^+$ subpopulation contains pluripotent stem cells with self-renewing potential. Furthermore the Ly-6A/E$^-$ Thy-1^{lo}Lin$^-$ (which express erythroid lineage markers) produced splenic colonies which were enriched for erythroid colonies, whereas colonies produced from Ly-6A/E$^-$ Thy-1^{lo}Lin$^-$ cells were enriched for all lineages including erythroid, thus the Ly-6A/E$^+$ cells are likely to be a homogeneous subpopulation of uncommitted stem cells.[33] The functional relevance of Ly-6A/E expression on Thy-1^{lo}Lin$^-$ cells is unknown although the relationship between the presence or absence of Ly-6A/E molecules on the cell surface and the differentiation status of stem cells might suggest a role in the mechanisms controlling the lineage commitment.

Ly-6 expression in the thymus

The generation of mature T cells from early precursors is a complex cascade of events that involves cellular proliferation, selection and deletion, and is represented phenotypically by the expression of markers that are associated with mature T cell function (CD4$^-$ CD8$^+$ CD3$^-$).[34] Ly-6 expression is uniquely regulated throughout T cell differentiation with the acquistion of double negative (CD4$^-$ CD8$^-$), double positive (CD4$^+$ CD8$^+$) and single positive (CD4$^+$ CD8$^-$, CD4$^-$ CD8$^+$) phenotypes (Table 2).

Ly-6A/E and TSA-1 expression on early thymic precursors

The sequence of differentiation events that link the lineage committed T cell precursors in the thymus with pluripotent bone marrow stem cells is not known although two lines of evidence suggest that Ly-6A/E expression is maintained throughout the process. First, the Ly-6A/E$^+$ Thy-1^{lo}Lin$^-$ subset from adult bone marrow contains cells with thymic precursor potential that can colonize an irradiated thymus on intravenous or intrathymic transfer.[33,34] Second, the earliest T cell precursor subpopulation in adult thymus which can reconstitute the lymphoid and dendritic elements of an irradiated thymus upon intrathymic transfer is also Ly-6A/E$^+$.[36,37] Together with the expression of other markers including CD4^{lo}, these observations suggest that the adult thymic Ly-6A/E$^+$ CD4^{lo} subset is the immediate progeny of thymus seeding Ly-6A/E$^+$ Thy-1^{lo}Lin$^-$ bone marrow stem cells.[38] Thus Ly-6A/E expression might conceivably be maintained throughout the transition process; if this was the case, then the phentoype of thymus seeding cells *in vivo* (irrespective of whether they are pluripotent stem cells or lymphoid committed precursors) would be Ly-6A/E$^+$.

The Ly-6A/E$^+$ Thy-1^{lo}Lin$^-$ bone marrow stem cells and Ly-6A/E$^+$ CD4^{lo} T cell precursors have differences in repopulation potential, with Ly-6A/E$^+$ CD4^{lo} subset having lost the capacity to generate myeloid and erythroid cells, and with a bias towards lymphoid development.[38] Thus, the Ly-6A/E$^+$ CD4^{lo} subset are committed to the differentiation of lymphoid lineage precursors. When Ly-6A/E$^+$ Thy-1^{lo}Lin$^-$ cells are directly injected into irradiated thymus lobes, the only difference observed in the expression of a number of phenotypic markers is the up-regulation of TSA-1; this precedes the cell surface expression of other markers that are associated with the later stages of thymopoiesis (i.e. CD8 and CD3).[38] Serological analysis of the Ly-6A/E$^+$ CD4^{lo} subset demonstrates that these cells also express TSA-1 (whereas pluripotent stem cells are Ly-6A/E$^+$ TSA-1$^-$) and accordingly, when the (TSA-1$^+$) subset is purified and transferred intrathymically to irradiated recipients, T cell precursor activity is observed as the appearance of lymphoid colonies but not myeloid or erythroid progenitors.[38] Thus the acquisition

Table 2 Ly-6A/E, ThB and TSA-1 expression relative to CD3,4,8 phenotypes during T cell differentiation

Thymic microenvironment	Differentiation status	Phenotype	Other markers	Selection	Ly-6A/E	TSA-1	ThB
CM/SC	T cell precursor	CD^{lo}CD8$^-$ CD3$^-$	Thy-1$^-$ H-2K$^-$ Pgp-1$^-$ HSA$^+$ c-kit$^+$		+ + +	+ +	
SC	DN	CD4$^-$ CD8$^-$ CD3$^-$	Pgp-1$^-$ IL-2R$^-$ HSA$^+$		+ +	+ +	?(+)
		CD4$^-$ CD8$^-$ CD3$^-$	Pgp-1$^-$ IL-2R$^-$ HSA^{+-}		+	− +	?(−)
		CD4$^-$ CD8$^-$ CD3$^-$	Pgp-1$^-$ IL-2R$^+$ HSA^{+-}		+ −	+ +	?(+)
		CD4$^-$ CD8$^-$ CD3$^-$	Pgp-1$^-$ IL-2R$^+$ HSA^{+-} TcRβ mRNA		+/−	+ +	?(+)
C	Transient SP	CD4^{lo}CD8$^-$ CD3$^-$			?	+ − +	?
		CD4$^-$ CD8^{lo}CD3$^-$			?	+ + +	?
	DP	CD4^{lo}CD8^{lo}CD3$^-$	HSA^{+-} TCRα/β mRNA		−	+ + +	?(+ + +)
		CD4^{hi}CD8^{hi}CD3-TcR^{lo}	HSA^{+-}	P/N	−	+ +	+ + +
		CD4^{hi}CD8^{hi}CD3-TcR$^{int/hi}$	HSA^{+-}	P/N	−	+/−	+ − +
		CD4^{hi}CD8^{hi}CD3-TcR^{hi}	HSA^{+-}	P/N	−	−	+ + +
CM	SP	CD4^{hi}CD8^{lo}CD3-TcR^{hi}	HSA$^+$ Qa-2$^+$	N (?)/A	+	−	+/−
		CD4^{lo}CD8^{hi}CD3-TcR^{hi}	HSA$^+$ Qa-2$^+$	N (?)/A	+	−	+/−
M	Thymic migrant	CD4^{hi}CD8$^-$ CD3-TcR^{hi}	HSA$^{+/-}$ QA-2$^-$		+	−	−
		CD4$^+$ CD8^{hi}CD3-TcR^{hi}	HSA$^{+/-}$ Qa-2$^+$		−	−	−
	Peripheral T cells	CD4$^-$ CD8$^+$				−	−
		CD4$^+$ CD8$^-$				−	−

CM, cortico-medullary; SC, subcapsular; C, cortex; M, medullary; DN, double negative; SP, single positive; DP, double positive; P, positive selection; N, negative selection; A, anergy; (+ + +), high expression; (−) no expression; (?), expression not reported.

of TSA-1 expression is phenotypically and perhaps functionally associated with lymphoid lineage commitment.

Ly-6A/E, ThB and TSA-1 expression in the cortical/subcapsular regions

Ly-6A/E expression is confined to connective tissue fibres and vasculature in the thymic subcapsular region and small isolated cellular clusters of $CD4^-CD8^-$, possibly including thymic epithelial nurse cells or macrophages in the thymic cortex.[24] Indeed, adoptive transfer of Ly-6A/E$^-$Thy-1^{lo}Lin$^-$ stem cells shows that Ly-6A/E expression decreases with IL-2R, Pgp-1 expression and increased HSA and $CD4^+CD8^-$ expression.[38] The Ly-6A/E phenotype on transient single positive cells, which are defined by the expression of CD4 or CD8 and represent an intermediate stage of thymic differentiation between the $CD4^-8^-$ and $CD4^+8^+$ cells (Table 2) has not been reported. TSA-1 molecules are expressed as Ly-6A/E$^-$Thy-1^{lo}Lin$^-$ stem cells, become lineage committed TSA-1$^+$CD4^{lo} precursors and approximately 90% of these cells are also Ly-6A/E$^-$.[38] The expression of TSA-1 is upregulated along with Thy-1, CD4 and CD8 such that 72.5% of $CD4^-CD8^-$ cells are TSA-1$^-$; this expression is presumably analogous to anti-Ly-6A.2 mAb staining observed on large blast cells in the thymic subcapsule.[24,38] There is TSA-1-specific staining in ribbon-like zones in the thymic cortex which is attributed to expression on 82.7% of $CD4^+CD8^-$ thymocytes.[24] The cell surface levels of TSA-1 are dramatically reduced with expression of CD3 molecules such that CD3$^-$TSA-1^{hi}, CD3^{lo} TSA-1^{lo} and CD3^{hi} TSA-1$^-$ subpopulations are observed; CD3$^{int/hi}$ cells express TSA-1 in a transient fashion and within the CD3$^{int/hi}$ population, TSA-1 expression is enriched in the CD3^{int} fraction.[39]

The origin of ThB expression during T cell ontogeny on $CD4^-CD8^-$ cells is not known although approximately 70% of total thymocytes express ThB and the absence of ThB expression on cortisone-treated thymus cells would suggest that expression is primarily in the thymic cortex.[40] Cortical ThB expression occurs on $CD4^+8^+$ thymocytes and the levels of ThB expression on this subset are unrelated to changes in TcR-CD3 expression.[39]

Ly-6A/E, ThB and TSA-1 expression in the thymic medulla

The cell surface expression of TSA-1 and ThB is lost with the transition of thymocytes from the cortex to the medulla. However, unlike TSA-1 expression, the loss of ThB appears to be associated with the down-regulation of both CD4 and CD8 expression (rather than CD3) because $CD4^+CD8^+TcR_{\alpha/\beta}{}^{hi}$ and $CD4^+CD8^+TcR_{\alpha/\beta}{}^{lo}$ cells are ThB^{hi} whereas $CD4^{int}CD8^{hi}TcR_{\alpha/\beta}{}^{hi}$, $CD4^-CD8^{hi}TcR_{\alpha/\beta}{}^{hi}$ and $CD4^{hi}CD8^{int}TcR_{\alpha/\beta}{}^{hi}$ and $CD4^{hi}CD8^-TcR_{\alpha/\beta}{}^{hi}$ cells are ThB^{lo}.[40] The lack of Ly-6A/E expression in the immature thymic regions is consistent with expression in cortisone treated mice, however, in the medulla Ly-6A/E molecules are expressed on blood vessels and stromal cells.[1,24] Ly-6A/E expression is on 30% of $CD4^+CD8^-$ cells and 10% of $CD4^-CD8^+$ cells, thus there is a bias for expression with CD4 T helper subsets.[24] It is not clear at which stage of single positive differentiation that ThB expression occurs although it is probably early (on $CD4^{hi}CD8^{lo}$ and $CD4^{lo}CD8^{hi}$ cells) because Ly-6A/E molecules are also observed on $CD4^-CD8^-$ cells which are presumably located in the corticomedullary region.[24]

The studies of Ly-6A/E, ThB and TSA-1, with reference to other differentiation antigens, suggest that the control of Ly-6 thymic expression may be of important functional significance because these molecules are intimately regulated with the transition of T cell precursors to peripheral T lymphocytes (Table 2). With regard to the putative accessory signalling role for Ly-6A/E molecules (discussed below) the down-regulation of Ly-6A/E expression on $CD4^-CD8^-$ cells prior to expression of TcR-CD3 and re-expression after selection events is clearly provocative. Thymocytes are presumably susceptible to selection events any time after which the TcR-CD3 complex is expressed[42] thus it may be advantageous to remove other accessory molecules (Ly-6A/E, IL-2R and Pgp-1)[38] before selection, thereby improving the quality of the signal that is transduced through the TcR-CD3 complex. After selection of a functional MHC restricted TcR-CD3 complex, Ly-6A/E molecules would be up-regulated to allow accessory signals to be transduced, with signals through the TcR-CD3 complex on peripheral T cells (Table 2). The down-regulation of TSA-1 expression is closely associated with the negative selection of $CD4^+CD8^+$ thymocytes because TSA-1 expression is inversely proportional to CD3 and is first down-regulated on CD3$^{int/hi}$ cells (Table 2). An interesting observation is that the $CD4^+CD8^-CD3^{hi}$ cells are devoid of autoreactive thymocytes[43–46] while the $CD4^+CD8^-CD3^{int}$ subset contains pre-deletion thymocytes.[44] It is not known whether the loss of TSA-1 expression on $CD4^-CD8^+CD3^{int/hi}$ cells *in vivo* is due to down-regulation or deletion of TSA-1$^+$ cells although *in vitro* studies would suggest down-regulation of expression.[47] The changes in ThB expression appear to be associated with the later events of thymocyte maturation and in particular with the transition of ($CD4^+CD8^-$) cells to the single positive phenotypes; there is no correlation with up-regulation of TcR-CD3 expression (Table 2) and the loss of ThB expression is associated with down-regulation

Table 3 RFD.2 analysis of Ly-6 related amino acid sequences

	ThB	TSA-1	Ly-6A/E	Ly-6C	Ly-6F	Ly-6G
ThB		2.6	3.9	3.9	3.9	3.3
TSA-1	2.8		3.3	3.0	3.0	4.3
Ly-6A/E	4.6	2.8		33.4	50 8	44.5
Ly-6C	3.8	3.2	30 1		32.8	27.1
Ly-6F	4 2	2 9	58.9	29.5		47.0
Ly-6G	3.1	4.7	43.8	27.1	49.1	

The optimal alignment between two Ly-6 sequences was calculated and expressed as the number of standard deviation (s.d.) units that the score is displaced from the mean scores of the two sequences after 100 random shuffling events; an alignment score of greater than or equal to 3 s.d. units was regarded as statistically significant.

of HSA molecules (Fig. 3). This finding is of interest because HSA molecules are down-regulated when $CD4^+ CD8^-$ cells are positively selected.[42]

Ly-6C thymic expression

The regulation of Ly-6C expression during T cell ontogeny is not known, although low levels of Ly-6C are detected in the thymus, in particular with animals treated with hydrocortisone and there is also expression on 6% of $CD4^- CD8^-$ immature thymocytes.[14,48,49] It is interesting to note that CTL effector cells generated from thymocytes are Ly-$6C^-$ whereas splenic Ly-6C expression is observed on $CD8^-$ T cells from athymic nude mice. These observations suggest that a distinct lineage of Ly-$6C^+$ cytotoxic T cells can be generated in the absence of thymic influence.[11] This suggestion is not unparalleled because peripheral T cells have been observed whose specificity is restricted to extrathymically expressed MHC molecules.[50]

ThB and TSA-1 expression on thymic stromal elements

Apart from expression on immature thymocytes, ThB and TSA-1 molecules are also expressed on clusters of thymic medullary epithelial cells which are likely counterparts of Hassals corpuscles.[51] These cells express surface markers which distinguish them from cortical epithelial cells, and two subsets are recognized according to expression of MHC antigens, although functionally they are poorly defined.[52]

Ly-6A/E, ThB and TSA-1 expression during B cell lymphopoiesis

TSA-1 molecules are expressed on 12% of bone marrow cells including a $B220^+$ (Ly-5) subpopulation and with serologically non-defined cells which do not express T cell, macrophage or granulocyte markers.[35] Ly-6A/E molecules are expressed on 7–10% of bone marrow cells (Table 1) including expression on approximately 5% of $B220^+$ cells which are also TSA-1^-.[35] It is not known whether this population represents pre-B or mature B cells although the observation that Ly-$6A/E^- Thy\text{-}1^{lo} Lin^-$ and Ly-$6A/E^+ TSA\text{-}1^+ CD4^{lo}$ cells are precursors of $B220^+$ cells might suggest that Ly-6A/E and TSA-1 expression is maintained during B cell ontogeny.[33] ThB is expressed on 25% of bone marrow cells (Table 1), including B cell precursors and mature B cells only, as studies with long term bone marrow cultures and B cell lines show that ThB^+ bone marrow cells are derived from earlier B-lineage cells and precede the production of B cells in the spleen.[53,54] In addition to differences in cellular morphology, two subsets of B cell precursors with the surface phenotype, $B220^+ sIg^- \mu_{cyt}$ (IgM heavy chain), are recognized, as a preferential association between ThB expression and small pre-B cells has been demonstrated on $(C57BL/6 \times DBA/2)F_1$ hybrid bone marrow cells, although there appears to be some overlapping expression on large pre-B cells.[55,56]

Ly-6A/E, Ly-6B, Ly-6C and Ly-6G expression on granulocytes and myelomonocytic lineages

Ly-6A/E expression is likely to be maintained during the sequence of events in which Ly-$6A/E^+ Thy\text{-}1^{lo} Lin^-$ cells are committed to the granulocyte or myelomonocytic lineages because Ly-6A/E expression is observed on precursors with $Mac\text{-}1^+ Ly\text{-}6G$ (or Gr-$1)^+$ phenotype.[33] Ly-6G expression is an accurate phenotypic marker of lineage commitment because $Ly\text{-}6G^-$ bone marrow cells contain precursor cells other than those of granulocyte or myeloid lineages. In fact, three subpopulations are defined according to Ly-6G expression: (i) $Ly\text{-}6G^{neg}$ cells are small, immature blasts that include lymphocytes and nucleated erythroid cells and comprise between 16 and 20% of total bone marrow; (ii) $Ly\text{-}6G^{lo}$ cells comprise 19–24% of total bone marrow with 55% immature blasts, 22% myelocytes and some neutrophils; and (iii) $Ly\text{-}6G^{hi}$ cells are large cells with 75% neutrophils. It is clear that the level of Ly-6G expression is inversely related to the ability of these populations to differentiate and proliferate in response to colony stimulating factors, because $Ly\text{-}6G^{neg}$ and $Ly\text{-}6G^{lo}$ populations show the greatest capacity to form colony forming unit cells in response to GM-CSF and little or no colony forming activity is observed in the $Ly\text{-}6G^{hi}$ population. Together with the observation that Ly-6G expression is induced on $Ly\text{-}6G^{neg}$ cells by GM-CSF and IL-3, these data suggest that the most immature population is $Ly\text{-}6G^{neg}$. In fact treatment of $Ly\text{-}6G^{neg}$ cells with saturating levels of IL-3, EPO, G-CSF and M-CSF demonstrates that this population has a potential to form cells in the granulocyte, macrophage, monocyte and erythroid lineages.[19]

Little is known about the expression of Ly-6B.2 molecules on progenitor cells. Although Ly-6B.2 expression is detected on at least 50% of bone marrow cells (Table 1), these molecules are not found on granulocyte/macrophage colony forming cells because pretreatment of bone marrow cells with anti-Ly-6B.2 mAb and complement does not reduce the capacity of these cells to form granulocyte/macrophage colonies on semi-solid agar.[10] Similarly, there is no evidence to support Ly-6C expression on haematopoietic stem cells although the reactivity of Ly-6C mAb with 50% of bone marrow cells (Table 1) suggests that Ly-6C is expressed on precursor cells, presumably of the myeloid or granulocyte lineages because Ly-6C expression is detected on bone marrow derived macrophages, eosinophils and neutrophils[16] and these cells do not constitute 50% of bone marrow cells.

Analysis of Ly-6 molecular structure

Ly-6 molecules are single chain, low molecular weight (12–20 kDa) glycoproteins which are connected to the cell surface by a phosphatidyl inositol anchor.[2] Gene cloning studies have shown that these proteins are defined by three distinct regions including: (i) a leader sequence with the aliphatic consensus, Leu/ValXxx(Ile/Val)LeuLeu(Val/Ala) (Val/Ala)LeuLeu; (ii) a peptide that forms a cysteine rich, hydrophilic domain with numerous potential signals for the attachment of O-linked, but not N-linked carbohy-

drate; and (iii) a-COOH terminal polypeptide which contains a consensus signal sequence (a pair of small residues (AlaAla) located 12 amino acids -NH_2 terminal to a hydrophobic domain (ValLeuLeuPheSerLeuSerScrValXxx-Leu GlnThrLeuLeu)), that is required for cleavage of the -COOH terminal peptide during phosphatidyl inositol anchor biosynthesis.

Comparison of murine Ly-6 amino acid sequences

The cell surface expression of Ly-6A/E, Ly-6C, Ly-6F, Ly-6G, ThB and TSA-1 molecules is characterized by a single unique domain containing cysteine residues which have remarkable conservation within the mature peptide (Fig. 2), suggesting that these residues are crucial for internal disulfide bonding. When cysteine residues are numbered in the context of placement in Ly-6A.2 amino acid sequence, equal spacing is observed between Cys^3 and Cys^6, Cys^6 and Cys^{15}, Cys^{15} and Cys^{20}, Cys^{20} and Cys^{27}, Cys^{27} and Cys^{48}, Cys^{48} and Cys^{52} and Cys^{73} and Cys^{78} in Ly-6A/E, Ly-6C, Ly-6F, Ly-6G, ThB and TSA-1 amino acid sequences, although ThB and TSA-1 sequences have different cysteine spacing between Cys^{15} and Cys^{20}, and Cys^{27} and Cys^{48}. Heterogeneity in cysteine spacing is observed between Cys^{52} and Cys^{72} for Ly-6A/E, Ly-6C, Ly-6F, Ly-6G, ThB and TSA-1 thus, this region may impart specific structural epitopes to these molecules.

There are other obvious regions of amino acid identity and homology that are common to Ly-6-related molecules and these residues are numbered in the context of Ly-6A.2 amino acid sequence (Fig. 2). At the -NH_2 terminus there is a LeuXxxCysXxxXxxCys motif such that residue 2 is charged (Met/Glu/Gln/Arg) and position 4 is an aromatic residue (Phe/Tyr/His). In addition, a highly conserved sequence is located at the Ly-6 -COOH terminus; this sequence (CysCysXxxXxxAsp LeuCysAsn) is found in all Ly-6 related molecules except that there are Asp^{76}/Ser^{76} and Leu^{77}/Phe^{77} substitutions in TSA-1. Furthermore, Asp^{24} and Pro^{53} residues are conserved in all murine Ly-6-related amino acid sequences except for ThB, which has Phe^{24} and Thr^{53} (Fig. 2). The amino acid sequences of allelic Ly-6A.2 and Ly-6E.1 are identical except for a Gly^{37}/Asp^{37} substitution (which is presumably responsible for the observed serological polymorphism) and an Ala^{80}/Val^{80} substitution within the -COOH terminal peptide.[2] Similarly, amino acid identity is observed for Ly-6C.2 and Ly-6C.1 although there is an Arg^{62}/Lys^{62} substitution (numbered according to Ly-6A.2 sequence) and a Val^{100}/Ile^{100} change in the -COOH terminal peptide.[2] There are regions of amino acid identity that are shared exclusively between Ly-6A/E and Ly-6C molecules including Tyr^7GlyValProxXxxGluThrSerCysPro and Ser^{38}GlnXxxArgLys(Val/Leu)Lys. Ly-6G molecules have Gly^8ValProxXxxGluThrSer in common with Ly-6A/E and Ly-6C molecules at the -NH_2 terminus and Ser^{23} AspGlyPhe and Leu^{47}CysLeuProIleCysPro sequences with Ly-6C and Ly-6A/E, respectively. An amino acid sequence, Asp^{37}SerArgLysXxxLys is conserved for Ly-6G and Ly-6C molecules and Met^{39} GluIleLeuGlyThr is common to Ly-6G and Ly-6A/E molecules. Ly-6F and

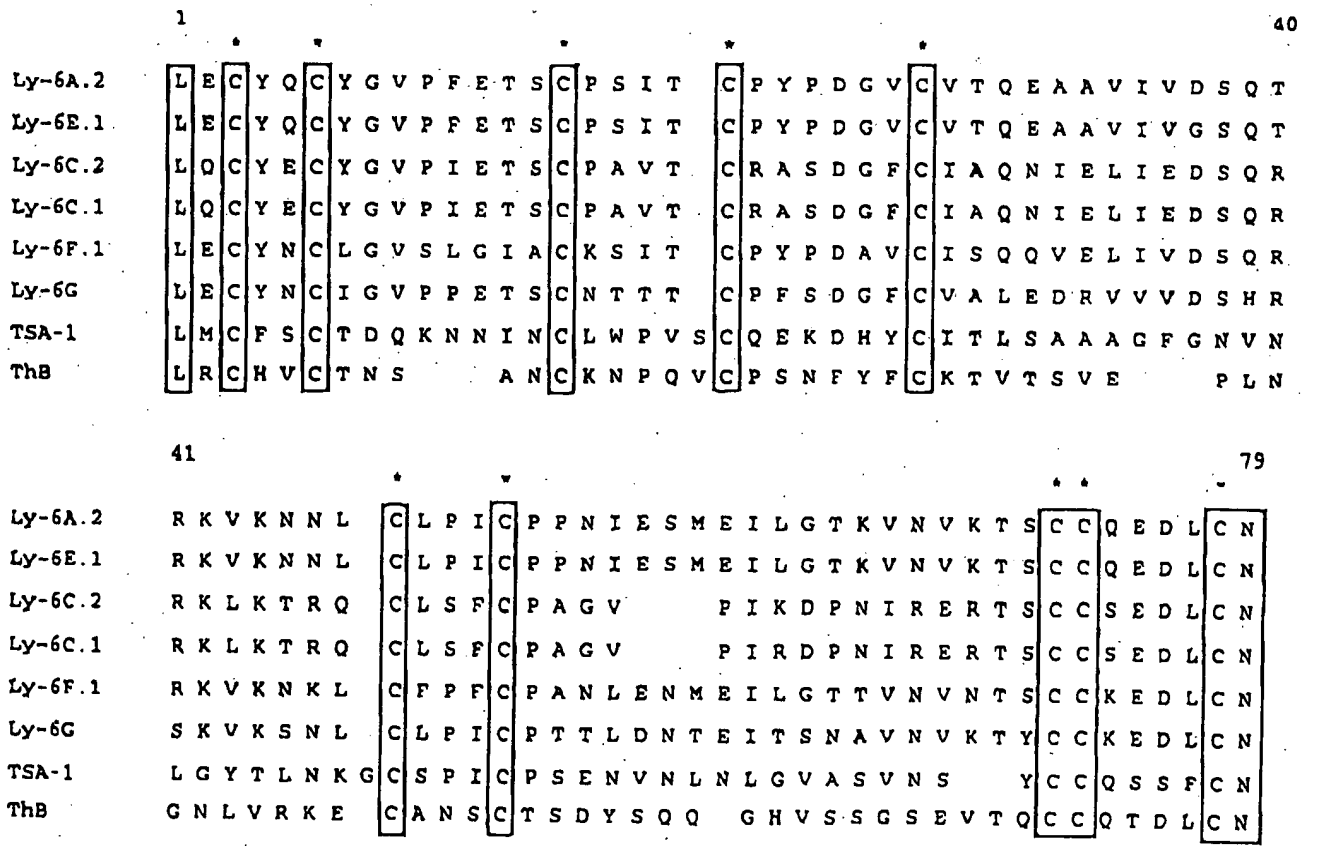

Figure 2 Amino acid sequence analysis of Ly-6 molecules. Amino acid sequences, from Leu^1 to Asn^{79} (numbered according to Ly-6A.2) were aligned by eye; the boxes show amino acid identity and (*) indicates conserved cysteine residues.

Ly-6G molecules both have $Val^{66}AsnVal$ (LysAsn)Thr in common with Ly-6A/E molecules. Apart from the aforementioned conserved -NH_2 and -COOH terminal sequences and aligned cysteine residues, TSA-1 molecules have few other regions of amino acid identity; $Pro^{50}IleCysPro$, $Val^{66}Asn$ and Asn^{47} are conserved with Ly-6A/E and Ly-6G molecules, Thr^{29}, $Ala^{32}Ala$ and Gln^{74} are conserved for Ly6A/E and TSA-1 and Ile^{28} is conserved between TSA-1 and Ly-6C. In addition to the conserved -NH_2 and -COOH terminal consensus sequences, ThB shares amino acid identity at Pro^{21} (numbered according to the Ly-6A.2 amino acid sequence) with Ly-6A/E, Ly-6F and Ly-6G, Phe^{26} are conserved in ThB, Ly-6C and Ly-6G, Thr^{29} is conserved between ThB and Ly-6A/E, and Leu^{43} and Glu^{68} are conserved between ThB and Ly-6C. Finally $Thr,^{7}$ $Asn,^{14}$ $Pro,^{18}$ $Asn,^{40}$ $Ser,^{54}$ Val^{63} and Ser^{65} are conserved between ThB and TSA-1 (Fig. 2).

The degree of amino acid identity between Ly-6 molecules has been quantitated using the RFD.2 program, in which the optimal alignment between two murine sequences was calculated and expressed as the number of standard deviation (s.d.) units that the score is displaced from the mean scores of the two sequences after 100 random shuffling events (Table 2). An alignment score of greater than or equal to three s.d. units is regarded as statistically significant. ThB and TSA-1 have the lowest alignment scores when compared with each other (2.6 and 2.8) and with other Ly-6-related molecules which suggests that *Thb* and *Tsa-1* genes diverged before the evolution of an *Ly-6* progenitor gene. Ly-6A.2 and Ly-6E.1 have the highest s.d. scores when compared with each other (67.3 and 65.8) as do Ly-6C.1 and Ly-6C.2 (63.8 and 62.6) which is consistent with the concept that Ly-6A.2/E.1 and Ly-6C.2/C.1 are allelic gene products.[2] Ly-6F.1 and Ly-6G molecules have high levels of amino acid identity with RFD.2 scores of 49.1 and 47.0, although Ly-6F has higher amino acid identity with Ly-6A.2 (58.9 and 50.8); Ly-6C has less amino acid identity with Ly-6G and Ly-6F (32.8 and 27.1).

Ly-6 gene superfamily

The observation of Ly-6-related amino acid sequence in squid glycoproteins supported the existence of a gene superfamily and Ly-6-like molecules have been characterized in the rat (RK 6, RK 10, RK 11 and RIP), human (CD59 and urokinase plasminogen activator receptor [uPAR]) and the snake neurotoxin/cytotoxin family (including Alb-SN, α-Bgt-LN and Nng-CYT).[57-61] The alignment of amino acid sequences for these molecules demonstrates a similarity with a consensus sequence derived from murine Ly-6 molecules (Fig. 3). In particular, there are 10 cysteine residues that are conserved in the amino acid sequences of RK 6, RK 10, RK 11, CD59 and uPAR (domain 2 and 3). The sequences for uPAR (domain 1) and Sgp-2 have variations of the murine Ly-6 like spacing of cysteine residues; uPAR lacks the seventh and eighth cysteine position and Sgp-2 lacks the eighth cysteine position. The snake toxins lack the third cysteine position and α-Bgt-LN and Nng-CYT lack the second cysteine position. However, the Alb-SN, α-Bgt-LN and Nng-CYT toxins and rat, human, squid and viral homologues all share the murine Ly-6-like -NH_2 terminal consensus (Leu/Ile)^{1}XxxCysXxxXxxCys and consensus sequence at the -COOH terminus (Fig. 3).

Apart from the conserved positions of cysteine residues and the -NH_2 and -COOH terminal consensus sequences, there are other regions of amino acid sequence that have been conserved and it is academic that phylogenetically distant members have fewer regions of identity and homology in common with murine Ly-6-related molecules compared with closer related species. Thus, by comparison of murine Ly-6-related amino acid sequences with phylogenetically different non-murine Ly-6, it is possible to determine which sequences impart structural integrity to specific murine Ly-6 molecules and which regions contribute to the general design of an Ly-6 related molecule.

The rat glycoproteins, RK 6, RK 10 and RK 11 share the highest level of amino acid identity with murine Ly-6 molecules. These molecules are more closely related to Ly-6A/E rather than Ly-6C; the mature protein sequence of RK 6, RK 10 and RK 11 has approximately 55% amino acid identity with Ly-6A/E and 35–40% identity with Ly-6C. There are three regions of amino acid identity which are conserved between murine Ly-6A.2 and rat RK 6, 10 and 11 molecules, which are described according to the position of residues in Ly-6A.2 amino acid sequence: (i) Thr^{19}CysProTyrXxxAsp GlyValCys XxxXxxGln; (ii) Lys^{42}ValLys; and (iii) Asn^{46}LeuCysLeuPro. There are fewer regions of homology between rat RK 6, 10, 11 and Ly-6C.2, according to the position of residues in the Ly-6C.2 sequence, including Thr^{19}CysXxxXxxXxxAsp Gly and Ser^{38}XxxArgArgLys (Fig. 3). Furthermore, the rat glycoproteins have amino acid identity with Ly-6A/E and not Ly-6C at Glu^{60}IleLeuGlyThrXxxVal (Fig. 3); this region could be related to Ly-6A/E structural integrity, or may interact with Ly-6A/E specific ligands. The region corresponding to Thr^{40} to Asn^{46} in Ly-6A.2 molecules is rich in basic residues which are also conserved in Ly-6C.2 and RK 6, 10 and 11. In particular there is a conserved Lys^{44} residue and three basic residues (Lys^{42}, Lys^{44}, Asn^{46}) are common within Ly-6A/E, RK 6, RK 10 and RK 11 and three basic residues (Arg^{40}, Arg^{41}, Lys^{42}) with Ly-6C. The net charge of this region is likely to be of critical functional significance.

There are regions of amino acid homology with murine Ly-6 molecules which have been conserved between phylogenetically distant species. In particular the Ly-6A.2 sequence from position 21 to 26 is homologous with non-murine Ly-6 molecules. Pro^{21} is conserved in Alb-SN and α-Bgt-LN, Pro^{23} is shared with uPAR (domain 2 and 3) and Asp^{24} and Gly^{25} in Ly-6A.2 are homologous with the charged residues (Asp, Glu and Lys) in the corresponding positions in the Alb-SN, α-Bgt-LN and Nng-CYT sequences. Furthermore, Val^{26} in Ly-6A.2 is homologous with an aliphatic residue (Leu) in the corresponding position in uPAR domain 1 and α-Bgt-LN and Nng-CYT sequences. Finally, Pro^{53} in Ly-6A.2 is conserved in all Ly-6-related sequences except for uPAR (domain 1 and 3), CD59 and Sgp-2 (Fig. 3). These regions of conserved

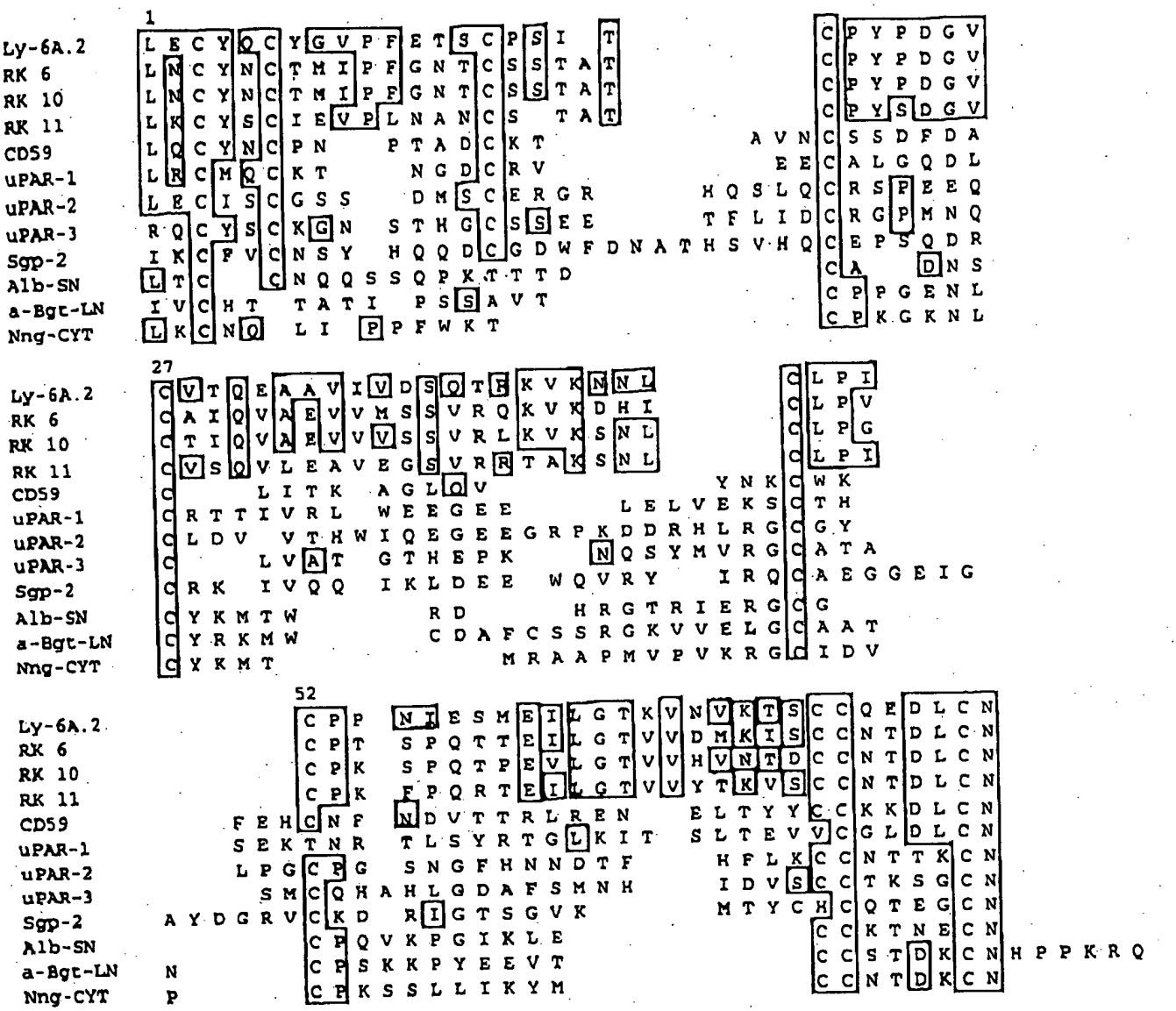

Figure 3 Amino acid sequence analysis of non-murine Ly-6 molecules. Amino acid sequences, from Leu[1] to Asn[79] (numbered according to Ly-6A.2) were aligned by eye; the boxes show amino acid identity.

amino acids are likely to contribute with the 10 cysteine residues to the general design of Ly-6 related molecules.

Although amino acid sequence is conserved with murine Ly-6, it is unlikely that these molecules are merely equivalents of the murine Ly-6 gene family. With the exception of RK 6, RK 10 and RK 11 molecules, which in addition to high amino acid identity are also encoded by a multiple gene family,[58] other Ly-6-related molecules are characteristically different to murine Ly-6. For example, the family of snake venoms is not expressed as cell surface glycoproteins, Sgp-2 is expressed in a phylogenetically distant species with no evidence of expression on immune cells and human uPAR contains multiple Ly-6-like domains whereas murine Ly-6 molecules are single domain glycoproteins. The inability of murine Ly-6 molecules to restrict complement mediated lysis and the absence of data suggesting a multiple gene family at the *CD59* locus suggests that CD59 is not the equivalent of murine Ly-6 molecules. The *CD59* gene maps to the short arm of Chr. 11 within the region *p14–p13*.[62] Although Chr. 11 has at least 30 loci that are associated with immune function it is interesting to note that only one of these gene products (MSK21) has similar biochemistry to CD59 and that the region of *p14–p13* with *CD59* is not syntenic with murine *Ly-6* on Chr. 15,[62] thus it is unlikely that *CD59* is the equivalent locus of murine *Ly-6*. Furthermore, the -NH_2 terminal amino acid sequence from a 21 kDa GPI anchored rat protein involved in complement restriction (RIP) is more homologous with CD59 than Ly-6A/E or Ly-6C.[59] Thus, with respect to the phylogenetic distance of rat and mouse, it is unlikely that CD59 is the equivalent of murine Ly-6 molecules. Nonetheless, the observation that Ly-6-like molecules are expressed in phylogenetically distant species suggests that there is a selective pressure to maintain the distinctive pattern of cysteine residues. Therefore, similar to the family of RK 6, RK 10 and RK 11 molecules, which is the analogue of the murine Ly-6, the equivalent of the murine Ly-6-related molecules is presumably conserved in other closely related species including humans.

Structural model of murine Ly-6 molecules

The first structural model for murine Ly-6-related molecules was extrapolated from the known crystal and solution structure of α-bungarotoxin, by virtue of the amino acid similarity and regions of predicted β-sheet that are shared between the snake toxins and murine Ly-6.[30] The primary sequence of α-bungarotoxin folds into the shape of a flat disk, with a globular core at one pole from which three anti-parallel loops emerge; this structural conformation is maintained by intra-chain disulfide bonds between first and fifth, second and third, fourth and sixth, seventh and eighth and ninth and tenth invariant cysteine residues.[63] The arrangement of these disulfide bonds is homologous to that determined for the related snake venom neurotoxins and cytotoxins and the same as the disulfide bonding pattern in uPAR.[64] The structural model of murine Ly-6-related molecules has been refined further by elucidation of CD59 solution structure.[65] Similar to α-bungarotoxin, CD59 folds into a flat disk (30 A × 30 A × 1.5 A) and consists of central triple stranded and double tranded β-sheets. However, there are some obvious differences between CD59 and α-bungarotoxin: (i) there is an extension of the first β-sheet finger between strands A and B in CD59 that includes two disulfide bonded cysteines that are conserved in murine Ly-6-related molecules but are absent from the snake toxin structure; (ii) the loop region between strands D and E has a helical structure in sCD59 and is shorter in the snake toxin model; and (iii) the loop between strands C and D is longer in the snake toxin structure than in CD59.

A hypothetical model for the structure of murine Ly-6-related molecules has been proposed by folding the murine Ly-6 peptides into a conformation that has been described in the structural models for the snake venom cytotoxin/neurotoxin family and sCD59[30,65] (Fig. 4). The Ly-6A.2 triple stranded β-sheet is likely to correspond with amino acids from Glu^{2} to Gln,[5] Ser^{17} to Thr^{19} and Val^{26} to Glu^{31} and the double stranded β-sheet contains residues Lys^{44} to Leu^{49} and Thr^{70} to Cys^{73}; the predicted helical structure in CD59 would correspond to Ly-6A.2 residues Asn^{55} to Leu^{62} (Fig. 4). *In toto* the globular core is presumably stabilized by disulfide bonding from invariant cysteine residues (excluding the second and third cysteine residues which are located between stands A and B) and two salt bridges, possibly, between the -NH_2 terminal α-amino group and Asp^{76}, and the -COOH terminal Asn^{79} and any one of the positive charged residues from Arg^{41} to Asn^{46} in Ly-6A.2. This region would also be stabilized by hydrophobic interactions from the aliphatic side chains corresponding to residues in Ly-6A.2 at positions, $Val^{,26}$ $Val^{,28}$ $Leu^{,47}$ $Leu^{,49}$ Ile^{51} and $Leu^{.77}$ The triple and double stranded β-sheets are presumably positioned proximal to the globular core and with the GPI anchor attached to asparagine in the globular core, loops one, two and three would protrude from the core and the exposed charged residues in Ly-6A.2 ($Glu^{,12}$ $Asp^{,37}$ Glu^{60} and Lys^{65}) could interact with a putative ligand.

Although the amino acid sequence of the uPAR and CD59 binding site for their ligands is unknown, a model of this site can be proposed by considering the physiological ligands for uPAR and CD59. The ligands for uPAR and CD59 are well characterized; uPAR interacts with the -NH_2 terminal fragment (amino terminal fragment or ATF) of uPA and CD59 binds the α-chain of C8 and the C9β domain.[66,67] The receptor binding sequence of ATF has been characterized and is homologous with a sequence described as the growth factor module; this sequence is required for the coupling of other factors (EGF and TGF-α) to their respective receptors.[68] Interestingly, the ATF and C9β domain share amino acid identity with each other across the growth factor module[69] (Fig. 5). The highest degree of homology between the ATF and C9β (from Cys^{13} to Cys^{19} and Cys^{31} to Cys^{33}) might provide the proper conformation for interaction with structurally similar CD59 and uPAR receptor sites and the lowest region of homology (between Val^{20} and Arg^{30}) might confer the receptor binding specificity. Thus, it is likely that the structural design of the binding sites on CD59 and uPAR are homologous. An insight to the general structure of the receptor amino acid sequence that interacts with the conserved regions of the growth factor module could be provided by chemically cross linking the Ly-6 ligand to the receptor, followed by protease digestion, immunoprecipitation and ligand elution, and microsequencing of the

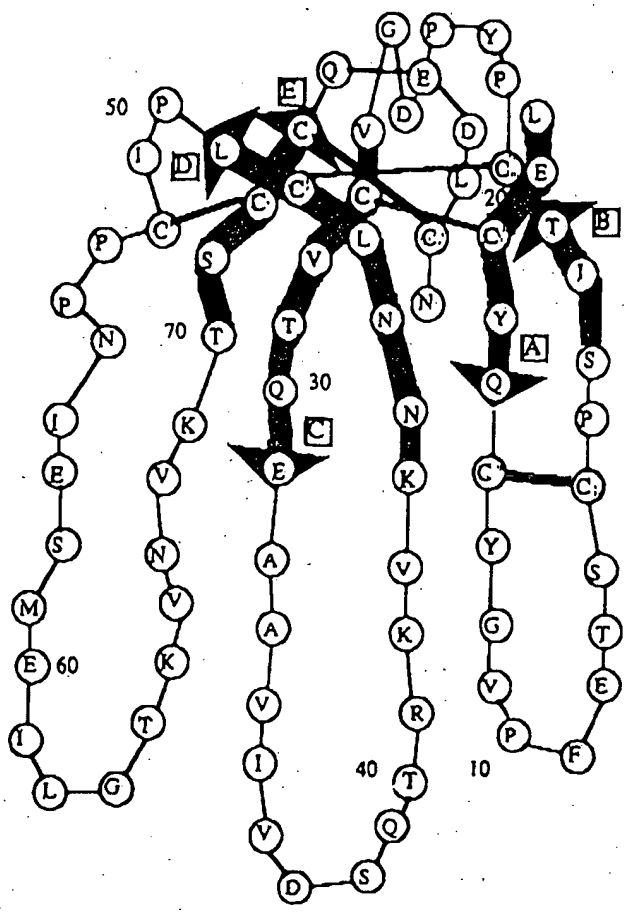

Figure 4 Structural model of Ly-6A.2 molecules. The Ly-6A.2 amino acid sequence was modelled on the crystal structure for α-bungarotoxin.[29] The conserved cysteine residues are represented by a stippled background and putative disulfide bonds are indicated by filled bars. The thick arrow indicates regions of predicted β-sheet structure.[65]

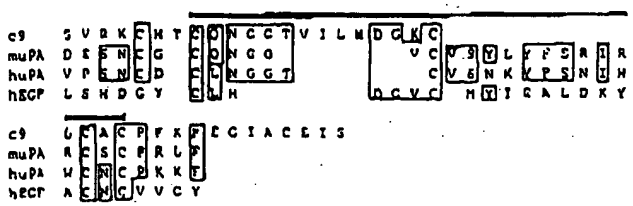

Figure 5 Alignment of amino acid sequence corresponding to the growth factor module of ligands for CD59, murine and human uPAR and human EGF receptor. The darkened line indicates the receptor binding region of the ligands and the residues within this region include c9; 491–508; muPA: 8–38; huPA 50–79; and huEGF: 8–37.

purified receptor-derived peptide. This approach has identifed residues of the EGF receptor that are in close proximity to the -NH_2 terminus of bound EGF.[70]

Although a ligand for murine Ly-6 molecules has not been identified, the observations of amino acid identity and, presumably, disulfide bonding which is common with CD59 and uPAR suggests that the receptor binding site on Ly-6 molecules might have a structural design that accommodates coupling with growth factor modules. Thus, in this model the specificity of Ly-6A/E, Ly-6C, Ly-6F, Ly-6G, ThB and TSA-1 and receptor-ligand coupling would be conferred by non-conserved amino acid residues and, similar to uPAR, the glycosylation status of the multiple potential O-linked glycosylation sites could play an integral role in the binding site conformation.[71]

Ly-6 expression in glycoprotein complexes

Ly-6 molecules appear to be expressed in multi-subunit complexes on the cell surface *in vivo*. A non-covalent association between ThB, Thy-1 and a 100 kDa T lineage surface protein (p100) can be detected with a naturally occurring anti-p100 mAb on the surface of CHAPS solubilized thymocyte membranes.[72] An observed resistance to PIPLC treatment suggests that the p100 molecule is a transmembrane protein. However, there is a loss of anti-p100 serological reactivity after removal of Thy-1 and ThB molecules by PIPLC treatment, suggesting that the p100 molecules are internalized subsequent to the cleavage of ThB and Thy-1 molecules. In the light of this unusual association, the role of p100 molecules in GPI mediated signal transduction is presently being investigated. When thymocyte membranes are cross-linked, anti-ThB mAb can immunoprecipitate ThB, Thy-1 and p100 and, similarly, ThB and p100 are co-precipitated with Thy-1 specific mAb. Other species that are resolved from cross-linked membranes with either mAb include those with molecular weights of 200, 45 and 12 kDa. The ThB-Thy-1-p100 interaction is stronger than the association between this complex and the 200, 45 and 12 kDa species.[72] In a separate study using anti-Thy-1 mAb and cross-linked T cell membranes, proteins with identical molecular weights are resolved and these have been identified as Thy-1, Ly-5 (a 200 kDa protein tyrosine phosphatase), MHC class I and ß2 microglobulin;[73] the expression of ThB on peripheral T cells. This complex is also immunoprecipitated without chemical cross-linking when cell membranes are solubilized in 1% digitonin. Most importantly, anti-ThB and Ly-5-specific mAb can immunoprecipitate a 200 kDa complex from [^{125}I]-labelled, cross-linked thymocyte cell membranes using either anti-ThB or anti-Ly-5 mAb. Two fragments of 16–18 kDa and 180 kDa are generated when the cross-linking reagent is cleaved and these fragments are identified as ThB and Ly-5 by limited proteolytic digestion.[74] In these experiments there was no evidence of species corresponding to p100, Thy-1 or the other loosely associated proteins described above; however, this may be due to the method of membrane solubilization. It is likely that tyrosine phosphatase activity can be co-precipitated with anti-ThB mAb as the precedence for this has already been established with anti-Thy-1 mAb.[73]

Ly-6 molecules have also been detected in large detergent-resistant glycolipid complexes with other GPI linked molecules and protein tyrosine kinases and their substrates. Both Ly-6A/E and Thy-1 are immunoprecipitated with p56^{lck} and two unidentified protein species (p35–40 kDa and pp80 kDa) from cell membranes lysed with mild (NP-40, CHAPs and Brij-58) detergents.[75] The structure of these complexes or 'GPI domains' is unknown although they do not dissociate when glycoproteins are removed by PIPLC digestion.[76] Thus, it is likely that the domains are maintained by hydrophobic interactions of membrane lipid moieties possibly including glycosphingolipids or cholesterol.[76,77] The association of these lipid complexes with intracellular protein tyrosine kinases is presumably with a myristoyl residue which could tether the intracellular molecules to the inner plane of the lipid membrane;[75,77] this anchorage would be analogous to the anchorage of glycoproteins to the external surface of the cell membrane. GPI domains are unlikely to be artifacts that are induced and held together by detergents because identical structures were observed with different detergents even when cells with Ly-6 GPI domains were lysed with different cell types containing different sets of GPI anchored proteins.[75] Furthermore, large detergent-resistant complexes containing Thy-1 with p53/p56^{lyn}[77] and Thy-1 with p59^{fyn}[78] have been detected in separate studies. The Ly-6-Thy-1-p56^{lck}, Thy-1-p53/p56^{lyn} and Thy-1-p50^{fyn} complexes have intrinsic phosphotransferase activity which is of interest with regard to the putative signalling function for Ly-6 molecules (and Thy-1).[77–79]

Characterization of the Ly-6 genetic region

The chromosomal location and fine structure of Ly-6-linked loci

The analysis of the segregation of *Ly-6* alleles with the *Xp-14*, *Gdc-1*, *Gpt-1* alleles (the latter three loci were known to map to Chr. 15) in NXSM RI lines demonstrates that *Ly-6* loci (*Ly-6A/E*, *Ly-6B*, *Ly-6C*, *Ly-6F* and

has been further refined to band 15E by *in situ* hybridization analysis.[81] The chromosomal mapping of *Thb* to Chr. 15 was first inferred by tight linkage of a gene regulating ThB expression with *Ly-6* and has been confirmed by interspecies back cross analysis.[21,82] Using a similar approach, two *Tsa-1* loci have been identified suggesting that there is one pseudogene and one structural gene in the mouse genome.[31] The functional *Tsa-1* gene maps to Chr. 15 with linkage order, *Myc-Tgn-Ly-6-Tsa-1-Sis* (thus *Tsa-1* also maps within band 15E) and (*Tsa-rs1*; for related sequence 1) maps to the middle region of Chr. 12 with linkage order *D12Nyu-1-Spnb-1-Fos*.

A physical map of the Ly-6 gene complex has been generated using field inversion gel electrophoresis, genomic library screening and two-dimensional gel DNA electrophoresis.[83] The map includes 1600 kb of genomic DNA and contains at least 18 distinct *Ly-6* related genes. A 630 kb Sac II species is the smallest fragment that contains all of the *Ly-6*-like species that hybridize to the Ly-6E.1 cDNA. There are twenty three *Ly-6*-related Hind III fragments that correspond to 15 distinct genes; these map within three clusters (A, B and C) of overlapping cosmid clones and a smaller non-overlapping cosmid clone, c63. Four additional *Ly-6*-related *Hind* III fragments that are detected by two-dimensional gel electrophoresis encode at least four distinct genes. The *Ly-6A/E* and *Ly-6C* genes map within separate cosmid clusters, A and B, respectively, and *Ly-6B* is understood to map within cluster C.[83] A comparison of the restriction maps for *Ly-6F* and *Ly-6G* to the *Ly-6* physical map suggests that *Ly-6F.1* probably maps to *Hind* III fragments, H22 and H12, in cluster C and *Ly-6G* corresponds to fragments H18 and H19 in cluster B.[30] The low *Thb* and *Tsa-1* nucleotide homology compared with the probes used in the fine mapping of murine *Ly-6* has prevented the assignment of *Thb* and *Tsa-1* to these clusters; thus it is not known whether *Thb* and *Tsa-1* maps to the 630 kb Sac II species or elsewhere within the 1600 kb fragment. Nonetheless, the ThB and TSA-1 cDNA will be useful tools for defining the position of other genes which have nucleotide sequence that is related to but separate from *Ly-6A/E*, *Ly-6C*, *Ly-6F* and *Ly-6G* genes. The *Ly-6* related genes have different orientation with respect to the direction of transcription and it is unclear what fractions represent functionally transcribed genes. It is of interest to note that some of the detected *Ly-6*-related genes lack leader sequences and are presumably pseudogenes which is consistent with the observation of other Ly-6-related pseudogenes including an Ly-6E.1-like gene with a 5 bp insertion in putative exon 4,[30] and a *Tsa-1-rs1* pseudogene.[31]

Analysis of Ly-6 gene organization

Ly-6A/E, *Ly-6C*, *Ly-6F*, *Ly-6G* and *Thb* have been characterized by the isolation of genomic clones and the organization of these genes is similar; the first exon encodes 5′ untranslated mRNA, together with a short 5′ segment of exon 2, the coding region of exon 2 encodes all but three amino acid residues of the putative leader sequence. The three -COOH terminal amino acid residues in the leader sequence are encoded by the 5′ region of exon 3 and the rest of the molecule, including signals for termination of translation and GPI anchor biosynthesis, are encoded by exon 4 (Fig. 6).[30,84–86]

Examination of the organization and nucleotide sequence of *Ly-6A/E*, *Ly-6C*, *Ly-6F*, *Ly-6G* and *Thb* show that they arose from a common ancestor gene by gene duplication and subsequent divergence. The nucleotide sequences for *Ly-6A/E* and *Ly 6C* show the highest level of homology with 78% nucleotide sequence identity; the greatest sequence similarity is across exon 2 (95%) and intron 3 is the least conserved and is longer in *Ly-6C*. There is 68% nucleotide sequence identity in the 5′ upstream regions including a B_1 repeat element that is located in the same area in *Ly-6A/E* and *Ly-6C*. Together the data demonstrate that *Ly-6A/E* and *Ly-6C* probably arose by duplication from a progenitor with the B_1 repeat element.[84] The nucleotide homologies of *Ly-6F* (exon four partial) and *Ly-6G* with *Ly-6A/E* are 84 and 82%, respectively, with homologies of greater than 75% for non-gapped sequences 5′ of exon 1 and for portions of the intron sequences. Thus, *Ly-6A/E*, *Ly-6C*, *Ly-6F* and *Ly-6G* are closely related at the level of nucleotide sequences. Although *Thb* has similar genomic organization, the *Thb* nucleotide sequence has the lowest homology with *Ly-6A/E*, *Ly-6C*, *Ly-6F* and *Ly-6G*, as demonstrated by the inability of ThB cDNA probes to detect *Ly-6* genes.[82] The evolutionary relationship of *Ly-6* and *Thb* genes has been clarified using phylogenetic tree construction and Ly-6A/E, Ly-6C, Ly-6F, Ly-6G and ThB amino acid sequences from Leu^1 to Asn^{79}; the inclusion of the TSA-1 amino acid sequence assumes that *Tsa-1* has *Ly-6*-like organization (Fig. 7). This analysis shows: (i) early divergence of the ThB amino acid sequence and progenitor sequence (node 2) from a common ancestor (node 1); (ii) divergence of the TSA-1 amino acid sequence before the evolution of the Ly-6 progenitor sequence at node 3;

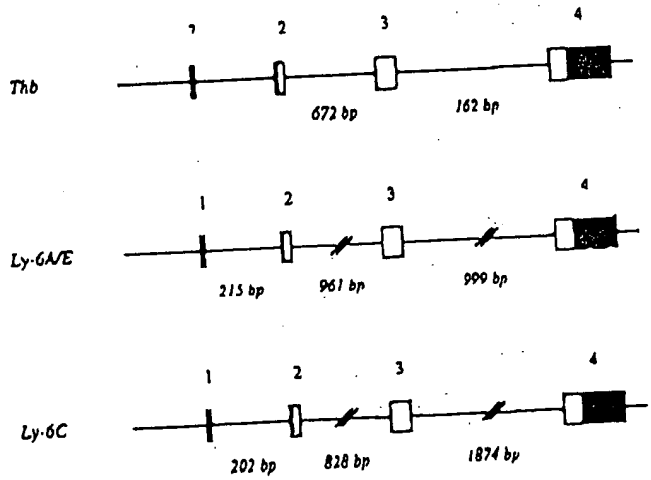

Figure 6 Comparison of *Thb*, *Ly-6A/E* and *Ly-6C* gene organization. The boxes represent exons and are numbered according to the exon structure of *Ly-6A/E* and *Ly-6C*; intron sizes are represented in bp. Coding sequence (□) and untranslated sequence (■) are indicated. The (?) represents uncharacterized 5′ untranslated ThB sequence.

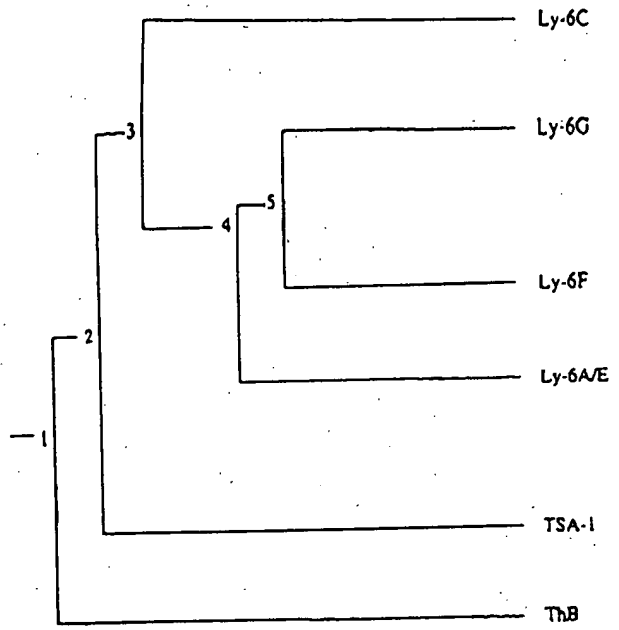

Figure 7 Phylogenetic tree constructed using the Clustal V program,[110] and the Phylip Protpars version 3.52c algorithim[111] and amino acid sequences for ThB, TSA-1, Ly-6A, Ly-6C, Ly-6F and Ly-6G from Leu[1] to Asn[73] (numbered according to ThB sequence).

and (iii) Ly-6G and Ly-6F amino acid sequences were related through a common ancestor (node 5) which diverged after the evolution of Ly-6A and Ly-6C amino acid sequences.

The similar organization of murine *Ly-6* genes is related to the arrangement of the 10 invariant cysteine residues within murine Ly-6 exons 3 and 4. The requirement of all 10 residues to form an Ly-6-like domain has prevented exon mixing between gene families and, in particular, the absence of an intron between the -COOH terminal end of the extracellular sequence and the signal sequence for GPI anchorage would prevent the Ly-6 domain from being duplicated and diversified in combination with exons encoding domains that are specific to other gene superfamilies.[87] Thus, Ly-6 consensus sequences have not been identified in 'mosaic' proteins with other non-Ly-6 domains.

Functional analysis of Ly-6 molecules

Although the serology, biochemistry and molecular biology of the murine Ly-6 gene family are reasonably understood, the functional role of these molecules *in vivo* is basically not known. The concept that murine Ly-6 molecules are involved in signal transduction stemmed from an observation that binding of anti-Ly-6 mAb can trigger cellular activation.[9] Thus, the focal point of the functional analysis has been the study of the molecular interactions involved in the signalling process in order to propose a model of Ly-6-mediated cellular activation.

Ly-6-mediated alternative pathway of T cell activation

The molecular events that lead to cellular activation are cell surface receptors with specific ligands. In the cellular activation of T lymphocytes, the ligation of the TcR/CD3 complex with MHC restricted peptide plays a key role in this process although there are other alternative pathways of activation including the receptor-ligand coupling of CD2 with CD48 or LFA-3 and CD28 with BB7 or CTLA-4. The cascade of events which follow include the generation of diacylglycerol and inositol triphosphate which are involved in the release of intracellular calcium stores ($[Ca^{2+}]_i$) and activation of protein kinase C. The later events of T cell activation are characterized by up-regulation of IL-2 receptor expression, cellular proliferation and production of IL-2. Using mAb specific for TcR-CD3 complex or CD2 and CD28, it is possible to mimic ligand binding and produce some of the spectrum of events that are characteristic of T cell activation; this phenomenon has been the basis for the putative role of Ly-6 molecules in the 'alternative pathway of T cell activation'.

The rat antibody D7, which recognizes a non-polymorphic framework epitope on Ly-6A.2/-6E.1 molecules, can induce early and late events of activation in T-T hybridomas, including increases in Ca^{2+} and IL-2 production, after cross-linking with anti-immunoglobulin or with Fc receptor-bearing antigen presenting cells. The activation of CD4 and CD8 resting T cells with D7 requires suboptimal concentration of PMA (a calcium ionophore which induces PKC).[6,88] Further to $[Ca^{2+}]_i$ increases and IL-2 production, IL-2 receptor expression and cellular proliferation are observed. In identical experimental conditions, the anti-Ly-6A.2 mAb, S8.106 and 34-11-3 have different proliferation potencies and compared with D7 activation is only observed when these mAb are cross-linked with anti-immunoglobulin.[88] The degree of heterogeneity that is associated with the ability of these mAb to induce T cell activation is not unexpected because D7 partially blocks S8.106 and 34-11-3 binding and presumably antibody affinity contributes to the potency of the stimulating mAb.

T cell activation is also observed when Ly-6C-specific mAb (6C3, 109-9-17, H9/25, HK 1.4 and 143-4-5) are cross-linked on the cell surface.[16,88,89] Similar to the activation potency of anti-Ly-6A.2 mAb, these mAb have different requirements to induce T cell activation, and the pathways of activation are often mAb-specific. The HK 1.4 mAb recognizes a non-polymorphic framework epitope and can induce the activation of cloned CTL in the absence of accessory cells, cross-linking of TcR or the addition of mitogen or PMA. Thus the potency of HK 1.4 is similar to 3A7 although these mAb bind to non-overlapping T cell populations. Interestingly, the HK 1.4 activation pathway is IL-2 independent because anti-IL-2 mAb do not block proliferation or lymphokine release. Furthermore, the stimulation of cloned CTL is not blocked by anti-TcR mAb and, in combination with HK 1.4 mAb, anti-TcR mAb have no additive stimulatory effect and the HK 1.4 mAb dramatically decreases the IL-2 driven proliferation of CTL.[16] Thus it is likely that HK 1.4 mAb activates cloned CTL via an IL-2, and possibly TcR-CD3 independent pathway. By comparison the 143-4-2 mAb induced activation of resting CD8 cells re-

quires the presence of sub-optimal doses of PMA or IL-2 expression of TcR-CD3 complex.[89]

The potential of ThB and TSA-1 molecules to induce thymocyte activation has been analysed by measuring changes in Ca^{2+} after cross-linking with 53–9.2 or MTS 35 mAb.[47,90] In these experiments there was no significant difference in $[Ca^{2+}]_i$ flux following cross-linking, although it is likely that the epitopes for 53–9.2 and MTS-35 mAb are not associated with activation. Indeed, if activation induced by Ly-6A/E and Ly-6C is physiologically significant, then ThB and TSA-1 are likely to have roles in cellular activation by virtue of the similarities between these molecules; the same argument would apply to the putative roles of Ly-6B, Ly-6F and Ly-6G.

A panel of TcR-CD3 mutant T cell hybridomas were analysed with the 3A7 (anti-Ly-6A.2) mAb to determine the role of the TcR-CD3 complex in Ly-6-mediated 'alternative pathway of T cell activation'.[91] The anti-Ly-6A.2 mAb (3A7) induces an extra-cellular derived Ca^{2+} flux in T-T hybridomas in the absence of cross-linking or other secondary signals, although resting T cells require sub-optimal doses of IL-2 for activation. This autocrine pathway of activation is driven by expression of IL-2 receptors and IL-2 secretion after cross-linking and is inhibited by anti-IL-2 receptor mAb. A CD3 loss mutant which lacks mRNA for TcR α- and β-chains and with normal cell surface levels of Ly-6 and Thy-1 did not secrete IL-2 when cross-linked with 3A7 mAb, although IL-2 production was observed when the parent $CD3^+$ line was stimulated with anti-Ly-6 or CD3. Furthermore, reconstitution of TcR α- and β-chains by transfection restored the capacity of the CD3 loss mutant to secrete IL-2 after CD3 or Ly-6 cross-linking.[92] These findings do not support the concept of an 'alternative pathway for Ly-6A.2 mediated T cell activation' because the activation events observed by Ly-6A.2 cross-linking are clearly dependent on TcR-CD3 expression. Thus, it would be of interest to determine the activation potential of other murine Ly-6 molecules on B cells, neutrophils, granulocytes and early T cell precursors that are clearly $TcR\text{-}CD3^-$. These experiments have been carried out, in part, by proliferation of $Thy\text{-}1^-$ bone marrow cells via Ly-6C cross-linking and the $[Ca^{2+}]_i$ flux and proliferation of murine B cells mediated by cross-linking anti-Ly-6A.2 mAb.[16,93] In the latter experiment, the $[Ca^{2+}]_i$ flux is derived from the intra-cellular Ca^{2+} pool in the absence of phosphatidyl inositol turnover which suggests that Ly-6A.2 mediated signalling pathway in B cells is independent to the pathway mediated by the cross-linking of surface immunoglobulin.

Role of Ly-6 in signal transduction through the TcR-CD3 complex

There is considerable evidence that suggests that Ly-6A.2 molecules are involved in the transduction of signals through the TcR-CD3 complex. Two phenotypically distinct classes of T-T hybridomas that are defective in Ly-6A.2 expression were demonstrated to have impaired function of the TcR-CD3 complex.[94] When stimulated with antigen and antigen presenting cells, with anti-TcR mAb coupled to Sepharose or with Con-A in the absence of antigen presenting cells, the Class I mutants (defective in Ly-6A.2 mRNA transcription but with normal expression of other T cell proteins) had markedly reduced IL-2 responses; this effect was related to the level of Ly-6A.2 expression because Class I mutants with higher levels of Ly-6A.2 cell surface expression secreted more IL-2 when activated. T-T hybridoma mutants with defective expression of GPI anchored molecules (Class II) showed reduction of IL-2 secretion although compared with Class I the difference was less significant. Although the mutations affected IL-2 production, the mutant lines had comparable responses to drug-induced receptor-like second signals which by-pass signals from the TcR-CD3 complex, from calcium ionophore and PMA stimulation.

These findings are paralleled by the use of antisense oligonucleotides to the 5′ end of Ly-6A.2 mRNA which decrease Ly-6A.2 expression on antigen primed lymph node T cells and T cell clones by 60–80%.[95] In this study, the inhibition of Ly-6A.2 expression prevented restimulation of antigen-primed T cells *in vitro* and blocked activation of resting T cells by lectins, anti-CD3 and anti Ly-6A.2 mAb. The decreased TcR-CD3 responsiveness in Ly-6A.2 antisense stable transfectants appears to be related to the cell surface levels of the α/β TcR heterodimer with a specific reduction in the level of TcR β-chain mRNA.[96] Thus, it is possible that Ly-6A.2 expression is important in the regulation of the TcR β-chain gene. Nonetheless, the inability to transduce signals through the TcR-CD3 complex is specific to the reduction in Ly-6A.2 expression because transfectants that are reconstituted with the TcR β-chain cDNA have decreased TcR-CD3 complex whereas normal responses to antigen, con-A and IL-2 are restored when cells are transfected with the Ly-6E.1 cDNA (which is antithetical to Ly-6A.2).

Tyrosine phosphorylation of multiple cellular substrates is observed within minutes of TcR engagement in normal T cells; these molecular events lead to the kinasing of 125, 105, 95, 70 and 64 kDa proteins.[97] When T cells were activated by cross-linking with anti-Ly-6A.2 mAb, the kinetics of substrate phosphorylation were slower and there was a notable absence of the 70 and 64 kDa species which suggests that cross-linking of Ly-6A.2 alone is insufficient to induce maximal tyrosine phosphorylation alone.[96] These results are consistent with the observations that TcR-CD3 loss mutants are not activated by cross-linking of Ly-6A.2 molecules alone.[91] Compared with the wild type T cell clone, the tyrosine phosphorylation of the 125, 105, 95, 70 and 64 kDa proteins is impaired in Ly-6A.2 antisense transfectants which is analogous to the reduced IL-2 responsiveness of mutants with significantly reduced levels of Ly-6A.2 molecules. Furthermore, the *in vitro* activity of the src-related *fyn* kinase is directly proportional to expression of Ly-6A.2 molecules; optimal *fyn* kinase activity and normal phosphorylation of cellular substrates (compared to the wild type T cell clone) is observed when Ly-6E.1 cDNA is reconstituted by transfection of Ly-6A.2 antisense clones, although activity is not restored by TcR β-chain cDNA transfection alone.[96] In these experiments, the kinase activity of *lck* was unaffected. Together, the data suggest that Ly-6A/E molecules

play a critical role in controlling optimal *fyn* kinase activity for the phosphorylation of proteins in the TcR-CD3 signal transduction pathway.

A fundamental problem with the concept that the absolute level of Ly-6A.2 expression is crucial for responsiveness through the TcR-CD3 complex is the observation that the baseline expression of Ly-6E.1 on resting T cells is less than 20% that of Ly-6A.2. The mutant T cell lines with defects in Ly-6A.2 transcription had a decrease in the levels of Ly-6A.2 expression ranging from 100% of normal (Class 1A mutants with defects in Ly-6A.2 mRNA transcription) to 40% of normal (Class IC mutants with defective expression of GPI anchored proteins); the magnitude of these decreases is analogous to the difference in the levels of Ly-6A.2 and Ly-6E.1 expression (Table 1). Nonetheless, Class IC mutants, with intermediate levels of Ly-6A.2 expression, have significantly lower responses to antigen compared with the parental cell with normal Ly-6A.2 expression. Furthermore, the reduction of Ly-6A.2 expression in antisense Ly-6A.2 transfectants was 60–80% (which is also within the differences of Ly-6A.2 and Ly-6E.1 expression) although real differences were observed in the proliferative response of naive and antigen primed lymph node T cells to con-A and IL-1, MHC restricted antigen and anti-CD3 mAb compared with non-transfected controls.[95] Finally, when T cells were treated with PIPLC and then stimulated with either con-A, calcium ionophore and PMA, or anti-CD3 mAb, the response to con-A was blocked by 90% whereas responses to anti-CD3 and ionophore and PMA where not affected.[98]

Apart from the results of cloning studies, which strongly suggest that Ly-6E.1 is the allele of Ly-6A.2, it is obvious that the mechanisms of signal transduction through Ly-6E.1, as described in these studies, are identical to Ly-6A.2 because signalling is restored when Ly-6A.2 antisense transfectants are reconstituted with Ly-6E.1 cDNA.[96] Thus, as an extension to this functional model, T cells derived from Ly-6.1 strains would be expected to show reduced responses to anti-CD3 mAb or con-A, compared with Ly-6.2 strains. Clearly, both strains respond to stimuli in an identical fashion. The concept that signal transduction through the TcR-CD3 complex is controlled by the relationship between Ly-6A.2 expression and optimal *fyn* activity, also raises the question of how signals are transduced in Ly-6A.2$^-$ T cells. The expression of Ly-6A.2 or Ly-6C.2 molecules is overlapping in Ly-6.2 strains (Table 1), thus TcR-CD3 signalling might be associated with the level of Ly-6C.2 expression on Ly-6A.2$^-$ T cells. This hypothesis is not supported by the finding that Ly-6A/E and Ly-6C mediated signalling via cross-linking is defective in A/J strain mice, although A/J T cells are triggered by cross-linking with anti-TcR or anti-CD3 mAb.[99] Furthermore, Ly-6E.1 is expressed on only 5% of lymph node and spleen cells, thus there would be T cells which are both Ly-6E.1$^-$ and Ly-6C.1$^-$ The increase in the proportion of Ly-6E.1$^-$ T cells in response to con-A can be completely inhibited by an anti-interferon-γ mAb, and these T cells are responsive to con-A. Thus, it is unlikely that mitogen induced enhancement of Ly-6E.1 expression is relevant to signal transduction through TcR-CD3

The model which proposes that Ly-6A.2 expression is essential for signal transduction through the TcR-CD3 complex is clearly at odds with the observations of functional TcR-CD3-mediated signalling with low or absent levels of Ly-6A.2. The restoration of functional TcR-CD3 signal transduction by transfection of Ly-6E.1 cDNA is the strongest evidence that points to a requirement for Ly-6A.2 expression, although it should be noted that these experiments were performed with T cell lines only. It is conceivable that other undefined cell surface glycoproteins make an important contribution to signal transduction on normal murine T cells and maintain the processing of signals through the TcR-CD3 complex when Ly-6A.2 expression is decreased, or when expression of Ly-6A.2 and other GPI anchored molecules (including Thy-1) are removed from the cell surface.

Role of the Ly-6 GPI anchor in signal transduction

A crucial event in signal transduction is the passage of information from the extracellular environment to the inside of the cell; most cell surface glycoproteins that are associated with this function have transmembrane and cytoplasmic domains which allow signals to be conducted across the cell membrane. The paradox of Ly-6-mediated signal transduction is that although these molecules could potentially collect signals from the extracellular environment, they clearly lack transmembrane and cytoplasmic regions which are required to transduce signals across the lipid bi-layer to the inside of the cell. Moreover, the phosphatidyl linkage that anchors Ly-6 to the cell surface appears to be an integral part of the signalling process because when Ly-6A.2 peptides are fused with transmembrane and hydrophobic domains, signals are not transduced by cross-linking with anti-Ly-6A.2 mAb.[100] The contribution of the GPI anchor towards Qa-2-mediated signal transduction was studied in an analogous experiment by the expression of hybrid molecules with transmembrane regions in transgenic mice.[101] Similarly, T cells from GPI linked Qa-2 molecules were activated with specific mAb but cells expresing Qa-2-transmembrane hybrids were not. In fact, the phosphatidyl inositol linkage is the only common characteristic which is shared between an array of otherwise unrelated cell surface glycoproteins which allow these molecules to transduce signals across the lipid bi-layer, following cross-linking. Thus, in this context, the cellular activation which is observed is attributed to 'GPI-mediated signalling'. The mechanisms of signal transduction by murine Ly-6 molecules and other GPI anchored proteins are not known although three models have been proposed to resolve this anomaly.[102]

First, a minor species of transmembrane counterparts which have identical external domains might be generated by alternative splicing of mRNA; these molecules would contribute wholly to signal transduction across the cell membrane. Although transmembrane and GPI anchored alternative isoforms of LFA-3 have been observed, the RNA, biochemical data and serological analysis of PIPLC treated cells do not support the notion that a transmem-

brane form of murine Ly-6 molecules exists.[103] Furthermore, the decreased response to con-A following PIPLC treatment, and the observation that Ly-6A.2-transmembrane hybrids do not transduce signals suggests that the GPI anchor, rather than a putative transmembrane counterpart, plays an integral role in cell activation.[98,100] Cleavage of the GPI anchor results in the generation of biologically active compounds including diacyl-glycerol and phosphatidic acid. Thus, it has been suggested that cross-linking the cell surface with mAb might activate phospholipase enzymes which could degrade the GPI anchor and produce secondary messengers which are ultimately involved in the activation of the cell. This process might occur after receptor internalization leading to diffusion of diacyl-glycerol into the cytoplasm and release of the peptide core to the cell surface by exocytosis. Indeed, cells are not activated if anti-Ly-6 mAb are attached to a solid matrix or plastic plates and Ly-6A.2 molecules are internalized during signal transduction by a distinct pathway which does not involve endocytosis or coated pits, although the time course of this process is not consistent with the early events of activation.[104,105] Conversely, the degradation process might occur outside the cell leading to extracellular shedding of murine Ly-6 molecules although T cell degradation of Ly-6 GPI anchors has not been observed and *in vitro* these digestions normally reduce T cell responses to con-A rather than increase them.[98]

The most likely scenario is that 'GPI-mediated signalling' occurs by interaction with presently undefined glycoprotein or glycolipid complexes. It is conceivable that clustering of Ly-6 molecules, mediated by mAb cross-linking, contributes to ultrastructural changes in the lipid bi-layer. Indeed, cross-linking of Thy-1 transmits a signal that causes polymerization of cytoskeletal components and their association with the membrane at the site of molecular clustering.[106] The changes in the cytoskeleton might contribute to the formation of specialized membrane domains, similar to the large-detergent resistant GPI domains which have been described previously.[75] These domains are sheets of lipid bi-layer associated with GPI anchors on the external plane and with intracellular kinases and other protein components covalently linked by a myristic acid to the inner plane. Associations between Ly-6A.2 and intracellular kinases have been demonstrated biochemically (i.e. $p56^{lck}$)[75] and functionally (i.e. $p59^{fyn}$)[96] and it is possible that other bridging proteinb components are also located in these domains which might transduce GPI mediated signals. Interestingly, unique lipid moieties including cholesterol and glycosphingolipids have been identified in large detergent resistant sheets and vesicles.[107] The detection of glycoshpingolipids is significant because these lipids and their catabolites modulate transmembrane signal transduction at the cell surface by influencing growth factor receptor-associated kinases and protein tyrosine kinase C;[108] thus there may be no requirement for bridging protein components between the GPI anchor and intracellular kinase for signal transduction.

Although 'GPI-mediated signalling' has been demonstrated by mAb cross-linking *in vitro*, it is unclear whether this process is an accurate representation of molecular events that occur *in vivo*. The results from stimulation experiments using glycoproteins fused with transmembrane segments and the observation of decreased responses to mitogens by PIPLC-treated T cells clearly identify the GPI anchor as the important contributing factor in signal transduction. However, the proteins which are recognized as co-stimulatory molecules *in vitro* are otherwise unrelated and many of these have been assigned very different functional roles *in vivo*. For example, CD59 and decay accelerating factor (DAF) are regulators of the complement cascade and uPAR plays a central role in the regulation of plasminogen-mediated extracellular proteolysis.[60,61] Together, the unrelated structure of CD59 and DAF, and the different physiological roles of CD59 and uPAR raise the question of whether these molecules are involved in signal transduction *in vivo*. Indeed, the observation that a non-stimulatory transmembrane molecule ($H\text{-}2D^b$) can activate T cells when fused with a GPI domain[101] after mAb cross-linking suggest that the nature of the peptide attached to the GPI anchor is irrelevant to signalling. However, it is unlikely that the Qa-2 *in vitro* signalling function is important for T cell activation *in vivo* because the transgenic line with the GPI derivative of $H\text{-}2D^b$ and transgenic mice which expressed Qa-2-transmembrane hybrid molecules were normal and immunodeficiency arising from impaired T cell function was not observed compared with parental lines.[102] These findings do not lend support to the notion that mAb-induced signal transduction is physiologically relevant for Qa-2 molecules and they also question the relevance of 'GPI-mediated signalling' models to the functional role of murine Ly-6 molecules *in vivo*.

The models which have been proposed to resolve the anomaly of signal transduction induced by cross-linking with anti-Ly-6 mAb are based on the concept that signal transduction through Ly-6A/E and Ly-6C is a unidirectional process. The fundamental problem with these models is that it is difficult to conceive how specific signals could be received and transduced along specific pathways. If the GPI anchor is the only factor which contributes to mAb induced signalling by release of anchor degradation products, then clearly specificity of signal transduction cannot be achieved. Conversely, if the GPI-associated Ly-6A/E and Ly-6C peptides are involved by interaction with transducing proteins then a large number of these proteins would be required to generate specificity; there is no evidence that signal transduction by glycosphingolipids can generate the diverse pathways of signal transduction that would be required.

A separate model of Ly-6-mediated cellular activation, which circumvents the problem of generating specific signal transduction across a lipid bi-layer, would propose that Ly-6 molecules induce signal transduction in accessory cells which ultimately leads to the activation of Ly-6 expressing cells. This model is in contrast to the unidirectional signalling which has been proposed in the models described above and is clearly at odds with the studies which show that there is no requirement for accessory cells to achieve cell activation.[9,88] Nonetheless, it is possible that bi-directional signalling occurs through mu-

rine Ly-6 molecules *in vivo*. A dual functional role for CD59 has been characterized; CD59 binds soluble factors C8 and C9 and also interacts with CD2.[109] CD59 and murine Ly-6 molecules are structurally related; thus it is possible that murine Ly-6 interacts with soluble factors, leading to signal transduction associated with the TcR-CD3 complex, and Ly-6 might induce signal transduction in other cells by interaction with their cell surface glycoproteins.

Conclusion

The studies described in this review have revealed many interesting properties of the murine Ly-6 family of molecules, in the context of tissue expression, molecular structure and function. Ly-6 molecules have tightly regulated patterns of expression on stem cells, lineage committed precursor cells and peripheral lymphoid tissues. These molecules share remarkable amino acid homology in terms of conserved cysteine residues, and the comparison of non-murine Ly-6 related amino acid sequences which have defined molecular structures suggest that the conserved cysteine residues are crucial for the conformation of a unqiue Ly-6-like protein domain. The observations of cellular activation from *in vitro* studies and the expression of Ly-6 molecules with protein phosphatases and kinases in membrane glycoprotein complexes suggest a functional role in signal transduction. However, many aspects of the Ly-6 family are not understood, and future studies in the context of: (i) the characterization of all transcribed *Ly-6*-linked genes and gene products; (ii) the relationship of the regulation of Ly-6 expression in haematopoietic and peripheral tissues to cell development and function; and (iii) the mechanisms of adhesion or ligand coupling interactions with other molecules will allow an insight into the functional role(s) of Ly-6 molecules *in vivo*.

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